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SHORT COMMUNICATION

Optimization of ultrasound-assisted extraction of camptothecin from *Camptotheca acuminata* seeds

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Abstract: Naturally occurring camptothecin (CPT) is an important source of chemotherapeutic agents. The extraction from *Camptotheca acuminata* is still the main approach to obtain CPT compared with total synthesis. In the present study, ultrasound-assisted extractions (UAE) of CPT from *C. acuminata* seeds with alkaline solutions were investigated and CPT yield were determined by High Performance Liquid Chromatography. The conditions of alkaline species and concentrations, extraction time, extraction temperature and ultrasonic power were optimized. Results show that both Na_3PO_4 and Na_2CO_3 solutions gain good extraction yields, whereas Na_3PO_4 solution has stronger basicity and need higher concentration than Na_2CO_3 solution does, thus aqueous Na_2CO_3 is more beneficial for the extraction. The optimal condition was ultrasonically extracted with 0.5% aqueous Na_2CO_3 at 50°C and ultrasonic power of 400 W for 60 min. Comparing with UAE with ethanol, the extraction with 0.5% Na_2CO_3 solution achieves higher yield. Moreover, aqueous Na_2CO_3 as a solvent has various advantages including non-toxicity, inflammable, non-corrosive and low cost, which ensure this UAE method is a superior method with high utilizing prospect.

Keywords: camptothecin; *Camptotheca acuminata*; ultrasound-assistant extraction

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Introduction

20(S)-Camptothecin (CPT), a pentacyclic alkaloid possessing high anti-tumor activity, was first isolated from a Chinese tree *Camptotheca acuminata* in 1966 (Wall et al. 1966). Unfortunately, research on CPT was interrupted for about ten years, for the carboxylated sodium, its water-soluble derivative used in clinical trials in early 1970s, is less effective and more toxic than CPT (Muggia et al. 1972; Moertel et al. 1972). Renewed interest in CPT is attributed to the elucidation of mechanism of cytotoxicity targeting to the topoisomerase I in 1985 (Hsiang et al. 1985). Since 1980s, CPT derivatives with high effectiveness, low toxicity and good water-solubility were developed as chemotherapeutic agent. Topotecan and irinotecan, two CPT derivatives were approved by the US Food and Drug Administration (FDA) in 1996 for the clinical treatment of refractory ovarian and colon cancer. Several other CPT analogues were investigated in various stages of clinical trials, including 9-AC, 9-NC, exatecan mesylate, and karenitecin (Carbonero et al. 2002; Hartmann et al. 2006; Teicher et al. 2008). Most of CPT derivatives are semi-synthesized from CPT and the production of CPT is still dependent on natural sources. CPT is often extracted by using organic solvents, such as methanol and ethanol (Fulzele et al. 2005; Zhang et al. 2007), mainly because the lactone form of CPT has better solubility in organic solvents. Previous studies show that the lactone ring of CPT is labile in aqueous solutions and easily transforms through reversible hydrolysis to the ring-opened carboxylate form (Fig. 1). This reaction is pH-dependent, and at equilibrium, the lactone form is predominant in acidic pH, whilst the ring-opened carboxylate form is favored under alkaline condition (Fassberg et al. 1992). We have reported a method to extract carboxylate form of CPT by diluted sodium hydroxide aq. under stirring and got CPT by acidifing the solution (Wang et al. 2000; 2001). This method need provide three times extraction and long time; moreover, various compositions can be dissolved in diluted sodium hydroxide aq. and precipitated by acidification, which result in a complex separation process and lower yield after purification.

Ultrasound-assisted extraction (UAE) method provides a high efficient contact between sample matrix and solvent. Acoustic cavitation, mechanical function and thermal function have direct effect on the efficiency of ultrasonic extraction (Romanik et al. 2007). Ultrasonic cavitation, the most significant factor, creates shear forces that break cell walls mechanically and improve material transfer (Ma et al. 2009). High frequency sound disrupts the plant cell wall thereby enhancing solvent penetration into the plant material and facilitating the release of extracts (Mason et al. 1996). Recently, UAE has attracted increasing attention due to its higher extraction efficiency with shorter extraction time compared to traditional methods, such as maceration extraction, soxhlet extraction and stirring extraction. Therefore, ultrasound is of undoubted interest as a promising method for the extraction of CPT. The aim of present work was to establish an UAE method for the extraction of CPT by alkaline solutions.

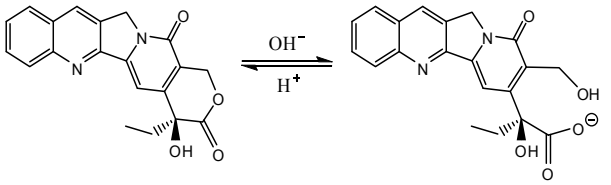

Fig. 1 Structures of lactone form and carboxylate form of camptothecin (CPT)

Materials and methods

Regent and chemicals

HPLC grade methanol used for the HPLC analysis was obtained from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was purified by MilliQ system (Millipore, Bedford, MA, USA). All other chemicals used in this study were of analytical grade from Sinopharm Chemical Reagent Co, Ltd. (Shanghai, China). Camptothecin (purity of 98%) was kindly gifted by Harbin Foran High-Tech development Ltd. (Harbin, Heilongjiang, China).

Plant materials

C. acuminata seeds were purchased from Jintang, Sichuan province, China, in February 2008. The seeds were ground in a FW100 Wileymill (Test Instrumental Co, Ltd, Tianjin, China), dried at 60°C and stored in a desiccator.

Instrument and analytical conditions

The UAE was performed in a KQ-500DB ultrasound cleaning bath (Ultrasound instrument Co., Ltd, Kunshan, Jiangsu, China). The working frequency and power were 40 kHz and 500 W, respectively. The internal dimensions of the ultrasonic cleaning bath were 50 cm × 30 cm × 15 cm with the volume of 22.5 L. A Multitemp III thermostatic circulator (GE Healthcare, Piscataway, NJ, USA) was equipped with the cleaning bath to give a steady water temperature in the cleaning bath.

CPT analysis was performed on a HPLC system with a 1525 pump, a 717 autosampler and a 2996 photodiode diarray detector (Waters, Milford, MA, USA). The samples were separated on a Luna C18 column (250 mm × 4.6 mm, 5μm) and a security guard (Phenomenex, Torrance, CA, USA). The mobile phases were water (A) and acetonitrile (B). The flow rate and injection volume were 1.2 mL/min and 20 μL, respectively. The time program of the gradient was B% linearly increasing from 20 to 40 in the first 10 min, and then decreasing from 40 to 20 in the following 6 min, and stable at 90 for 4 min. At 20 min, it's ready for next injection. The typical retention time of Camptothecin CPT was 10.42 min. The chromatographic peaks of CPT in different solvents were confirmed by comparing their retention time and UV spectra with CPT standard. Peak area was integrated at 254 nm and quantification was carried out using external standard method (Fig. 2).

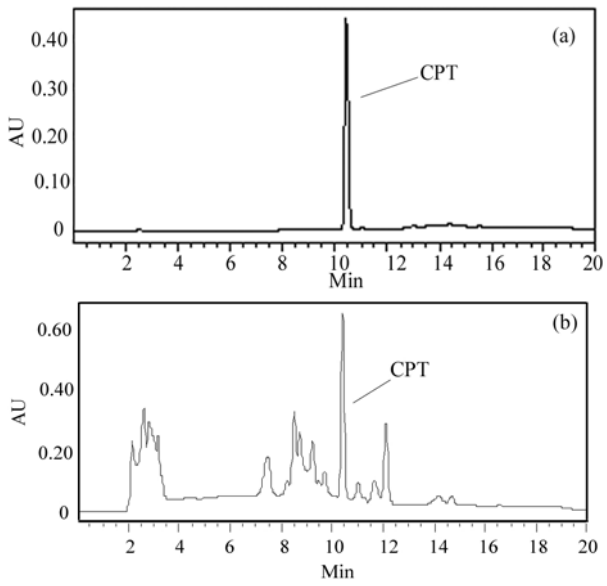

Fig. 2 The chromatograms of camptothecin (CPT) standard (a) and sample (b)

Sample preparation

1 g seed powder was loaded into a 50-mL conical centrifuge tube, and 20 mL of selected alkaline solution was then added. After votexing for 1 min, UAE was performed under certain conditions. After extraction, the extract was centrifuged at 3,500 ×*g* for 15 min to precipitate the residue of plant materials and the supernatant stored for HPLC analysis.

To test CPT concentration, 500 μL extract was transferred into a 2-mL microcentrifuge tube, and then 10% HCl was added for neutralization, followed by the addition of 1 mL methanol and mixed for several minutes. Above solutions were centrifuged at

12,000 ×*g* for 15 min, the supernatant was ready for HPLC analysis. All samples were prepared and analyzed in triplicate.

Results and discussion

Alkine species and concentrations were key factors affecting the extraction yield of CPT in our preliminary experiment. In the present study, strong, moderate and weak inorganic alkaline aqueous solutions (NaOH, Na_3PO_4, Na_2CO_3 and $NaHCO_3$ solutions at the concentrations of 0.1%, 0.5%, 1%, 2%, and 5%) were selected. The effects of various alkaline aqueous solutions on CPT yield were investigated. Using NaOH solution, the optimal yield was 0.073% at the concentration of 0.5%, whereas the yield significantly decreased to below 0.03% when NaOH concentration up to 2%. The extraction with aqueous Na_2CO_3 and Na_3PO_4 showed similar variation in CPT yield. Na_2CO_3 solution could achieve the yield over 0.08% at concentration of 0.5%, whereas in Na_3PO_4 solution, the yield was increased from 0.059% to 0.084% with the increase in alkaline concentration from 0.1% to 2%. Under $NaHCO_3$ solution, CPT yield was always lower than 0.05%. In summary, the CPT yield under Na_3PO_4 and Na_2CO_3 solutions were higher than that under the other solutions. In addition, the carbonate ion will be transformed into carbon dioxide when acidification was performed, it makes for the purification. Therefore aqueous Na_2CO_3 was the optimal solvent for the extraction of CPT, the concentration was selected 0.5% (Fig. 3).

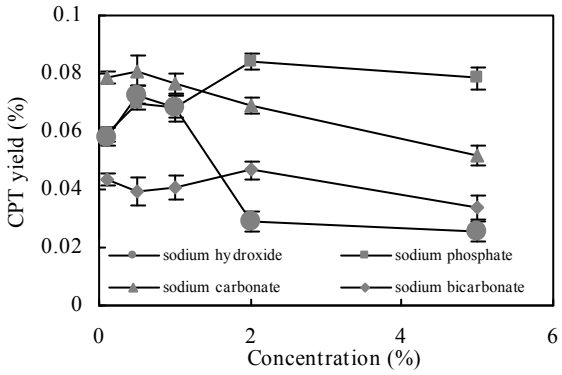

Fig. 3 Effects of different kinds of alkaline solutions and concentrations on CPT production. Values represent the mean and standard error of the yield of CPT between the triplicate sample preparations.

To optimize the extraction time, the seed powder was ultrasonically extracted by 0.5% Na_2CO_3 solution at the ultrasound power of 200 W and temperature of 30°C, respectively. The result showed that the extraction time was crucial to the increase of CPT production. When the extraction time was prolonged from 10 min to 60 min, the yield of CPT was increased due to the mass transfer of carboxylated sodium of CPT from cellular material to alkaline solution by diffusion and osmosis. Further extended extraction time, the yield started to decrease. It was reported that CPT is instable under the treatment of longer ultrasonic extraction time (>60 min) (Fulzele et al. 2005). Our result indicated that longer ultrasonic time might also have the same influence on the carboxylated form of CPT (Fig. 4).

To optimize the extraction temperature, the seed powder was ultrasonically extracted by 0.5% Na_2CO_3 solution for 60 min at different extraction temperature. Ultrasonic temperature did not significantly affect the yield of CPT at the range of 20–60°C. The yield showed an increasing tendency with the increase in temperature from 20°C to 50°C (Fig. 5). Thus, 50°C was selected as the optimum ultrasonic extraction temperature.

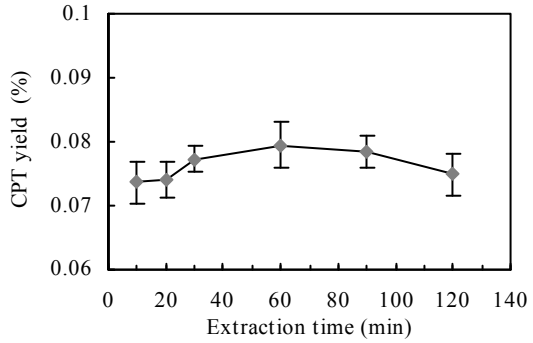

Fig. 4 Effect of the extraction time on CPT production. Values represent the mean and standard error of the yield of CPT between the triplicate sample preparations.

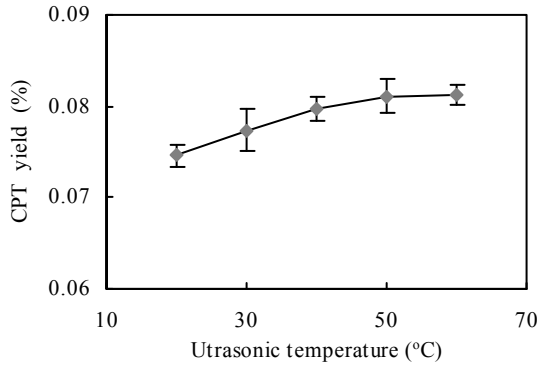

Fig. 5 Effect of the ultrasonic extraction temperature on CPT production. Values represent the mean and standard error of the yield of CPT between the triplicate sample preparations.

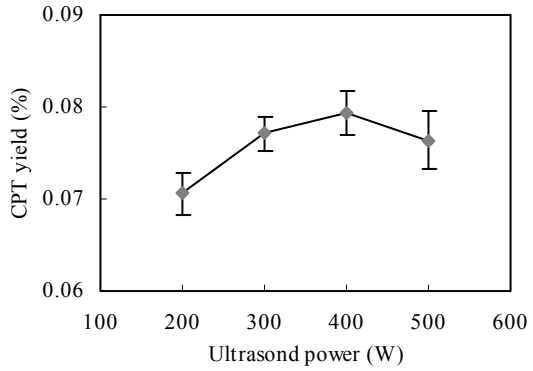

Fig. 6 Effect of the ultrasonic extraction power on CPT production. Values represent the mean and standard error of the yield of CPT between the triplicate sample preparations.

The effect of ultrasonic power on the yield of CPT was shown in Fig. 6. When ultrasonic power was increased from 200 W to 400 W, positive effect on the CPT yield was observed, whereas the extraction power was further increased over 400 W, the yield started to decrease, which may indicate that carboxylated sodium of CPT was unstable undergo high ultrasonic power treatment.

Based on the results above, the optimal conditions for ultrasonic extraction of the seed CPT were: 0.5% aqueous Na_2CO_3 at 50°C and ultrasonic power of 400 W for 60 min.

In previous studies, CPT was extracted mainly through traditional extraction method or UAE with ethanol. The present study carried out the CPT extraction by UAE with Na_2CO_3 solution. To validate the efficiency of UAE with Na_2CO_3 solution, we compared the CPT yield under UAE with 0.5% Na_2CO_3 solution and with ethanol at concentrations of 30%, 60%, and 100% (V/V) (Fig. 7). Results showed that CPT yield was the highest under UAE with 0.5% Na_2CO_3 for 60 min, followed by with 60% ethanol (p<0.05) and the yield was low under UAE with 30% or 100% ethanol (Fig. 7). Na_2CO_3 solution is non-toxic, inflammable, non-corrosive, and low-cost, which makes it more advantageous than ethanol as an extraction solvent and more suitable for the chemical and industrial production. Hence, the UAE with 0.5% Na_2CO_3 solution is a superior method with high utilizing prospect.

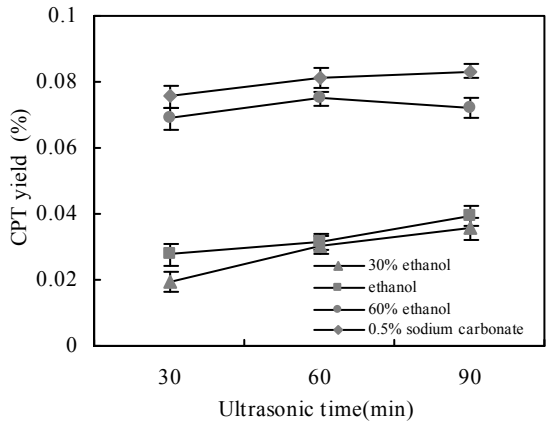

Fig. 7 Variation of CPT yield through ultrasound-assisted extraction with 0.5% Na_2CO_3 and ethanol. Values represent the mean and standard error of the yield of CPT between the triplicate sample preparations.

Conclusion

The UAE of CPT from *C. acuminata* seeds with alkaline solutions was investigated. The conditions of alkaline species and concentrations, extraction time, extraction temperature and ultrasonic power were optimized, and the results displayed that both Na_3PO_4 and Na_2CO_3 solution could gain good extraction yields, whereas Na_3PO_4 solution is a stronger base and need higher concentration than Na_2CO_3 solution, aqueous Na_2CO_3 was more beneficial for the extraction. The optimal conditions for the seed CPT extraction were ultrasonically extracted with 0.5% aqueous Na_2CO_3 at 50ºC and ultrasonic power of 400 W for 60 min. Comparing with UAE with ethanol, the extraction with 0.5% Na_2CO_3 solution achieved higher yield with significant difference. Moreover, aqueous Na_2CO_3 as a solvent has all advantages including non-toxicity, inflammable, non-corrosive and low cost; this UAE method is a superior method with high utilizing prospect.

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